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DESCRIPTION

Cdc7-ASK KINASE COMPLEX, SUBSTRATES OF THE KINASE COMPLEX,  
SPECIFIC ANTIBODIES TO THE SUBSTRATES, AND SCREENING METHODS  
5 USING THE SAME TO SCREEN FOR COMPOUNDS COMPRISING Cdc7-ASK  
KINASE INHIBITORY ABILITY

Technical Field

10 The present invention relates to methods for measuring the phosphorylation activity of Cdc7-ASK kinase complex.

Background Art

15 During cell growth, cells repeat cyclic processes whereby they replicate their genomic DNA, distribute this DNA equally among daughter cells, and then divide. This type of cycle is referred to as a cell cycle. The cell cycle can be divided into the G1 phase (DNA synthesis preparatory phase), S phase (DNA synthesis phase), G2 phase (mitosis preparatory phase), and M phase (mitotic phase). Cells divide by going through each stage  
20 in order. The progress of the cell cycle is finely regulated by numerous molecules, controlling unnecessary cell growth. To date, a number of molecules involved in cell cycle progression have been cloned and functionally analyzed. Abnormalities in molecules involved in cell cycle progression have been reported  
25 in numerous cancers, and can be considered possible causes of carcinogenesis.

To date, a variety of anti-cancer agents and anticancer agents have been developed. Much abnormal cancer cell growth is recognized as originating from abnormal cell cycle progression.  
30 In line with this, anticancer agents that targeting factors which regulate cell cycle progression are being vigorously developed. In particular, the function of Cdk-Cyclin, which has been most researched as a target for anticancer agents, has been considerably analyzed. Numerous candidate molecules for  
35 anticancer agents that target Cdk-Cyclin have already been reported.

Cdk is a family of molecules with numerous structural similarities, and each of these molecules forms complexes with various cyclin molecules specific to the cell cycle, thus regulating the various stages of the cell cycle. At present, particular molecules that specifically inhibit only Cdk-Cyclin have not yet been identified from among these structurally similar molecules. In other words, obtaining a molecule that specifically and selectively acts on cancer cells by targeting Cdk-Cyclin has been difficult. In addition, although numerous anticancer agents have been found that target regulatory factors in cell division or the G1 (stationary) phase, few agents have been discovered that target the process of DNA replication, another important regulatory point of cell growth.

A serine/threonine kinase different from Cdk-Cyclin has been found to play an important role at the S phase initiation point (the transition from G1 to S). Namely, in variant line Cdc7, which was isolated as a line with a variant cell division cycle (J. Mol. Biol., 59:183-194, 1971), the Cdc7 protein kinase has been demonstrated to function just prior to the initiation of chromosomal DNA replication, and to be necessary for activation of each origin of replication throughout the S phase (Mol. Cell. Biol., 6:1590-1598, 1986; Genes Dev., 15:480-490, 1998; and Genes Dev., 15:491-501, 1998). In addition, yeast Cdc7 kinase activity is known to be dependent on regulatory subunit, Dbf4 (Genetics, 131:21-29, 1992; and Mol. Cell. Biol., 13:2899-2908, 1993).

Expression of Dbf4 in yeast is cyclical, and regulated at both the transcription and post-translation levels (Exp. Cell Res., 180:419-428, 1989). At least some of the increase in Cdc7 kinase activity during the G1-S transition phase is explained by an increase in expression of Dbf4 during the late G1 phase (Mol. Cell. Biol., 13:2899-2908, 1993; and Exp. Cell Res., 180:419-428, 1989). Moreover, since Dbf4 interacts with the origins of replication in cells (Science, 265:1243-1246, 1994), Cdc7 is thought to trigger S phase initiation by directly activating the replication apparatus formed on an origin of replication.

The present inventors succeeded in isolating H37 protein, which is a human homolog of yeast Dbf4, and DNA encoding it (Unexamined Published Japanese Patent Application No. (JP-A) 2000-135090, J. Biol. Chem., Vol. 275, No. 37, 29042-29052, 2000). H37 is an important regulatory factor that governs the switching on and off of DNA replication initiation. H37 was subsequently named a human Activator of S phase Kinase (ASK).

ASK is a unique gene in human chromosomes, and its function is essential for chromosome replication. Based on the results of developmental engineering genetic analyses using mice, Cdc7 function was verified as being essential at the cellular and animal levels. Cdc7 encodes a kinase catalyst subunit, while ASK encodes each of the active subunits. The two form a complex, comprising an active kinase. The activity of Cdc7-ASK kinase is strictly regulated by the cell cycle, and increases in the S phase when DNA replication occurs. Although this kinase activity is maintained at a high level throughout the S phase, it is no longer detected from the M phase to the G1 phase. Regulation of this activity mainly depends on fluctuations in the level of ASK protein expression. Namely, the amount of ASK protein fluctuates during the cell cycle, and as a result, the activity of human Cdc7 kinase, which is regulated by being bound to ASK, also fluctuates during the cell cycle.

In addition, the expression level and activity of human Cdc7-ASK is typically observed to increase in actively growing cancer cells. In such cancer cells, the activity of human Cdc7 kinase activity is thought to increase with increasing expression of ASK protein. Of even further interest, the present inventors found that the expression level of ASK increases in response to increases in the growth ability of various cultured cancer cells.

Subsequent research by the present inventors demonstrated that the specific substrate phosphorylated by Cdc7-ASK complex is a minichromosome maintenance (MCM) complex (heterohexamer), essential for the initiation of chromosome replication (J. Biol. Chem., Vol. 275, No. 37, 29042-29052, 2000). On the basis of

this finding, the present inventors advocate a model in which Cdc7-ASK phosphorylates the MCM present in the replication origin complex, and as a result, induces reconfiguration of the subunit structure, causing the double-stranded DNA split that is essential for replication initiation.

On the basis of this, phosphorylation of a substrate protein by the Cdc7-ASK complex is recognized as being an important phenomenon in regulating the growth of cancer cells. However, many aspects of this phosphorylation mechanism remain to be elucidated.

#### Disclosure of the Invention

An object of the present invention is to clarify the mechanism of substrate protein phosphorylation by the Cdc7-ASK complex, and to provide methods for evaluating its activity. In addition, based on these evaluation methods, an object of the present invention is to provide methods for measuring the influence of Cdc7-ASK complex test compounds on the substrate phosphorylation effect. Another object of the present invention is to provide substrates, antibodies, or Cdc7-ASK complexes useful for these methods.

The present inventors considered the Cdc7-ASK complex to be a novel and important anti-cancer agent target; that is, they thought that inhibitors that could inhibit the phosphorylation effect of the Cdc7-ASK complex could selectively regulate cancer cell growth, and would be candidates for effective anticancer agents.

In order to evaluate the action of inhibiting the Cdc7-ASK complex phosphorylation effect, it was first essential to establish methods to evaluate this effect. To achieve this, the present inventors first clarified the mechanism used by the Cdc7-ASK complex to phosphorylate substrate proteins. On the basis of this finding, the present inventors confirmed that the kinase activity of the Cdc7-ASK complex was measurable, thereby completing the present invention.

The present inventors then discovered that the influence

of test compounds on the Cdc7-ASK complex's substrate protein phosphorylation function can be evaluated by applying these methods to measure kinase activity. On the basis of this finding, the present inventors established methods for evaluating the inhibitory (or promotional) effect of test compounds on Cdc7-ASK kinase, and methods of screening for compounds comprising such effects, thereby completing the present invention.

Moreover, while establishing these methods, the present inventors discovered novel substrate compounds, antibodies, Cdc7-ASK complexes or so on useful to these methods, thereby completing the present invention. Namely, the present invention relates to methods for measuring the kinase activity of the Cdc7-ASK complex, as below. Alternatively, the present invention relates to methods for evaluating the effect of test compounds on the kinase activity of the Cdc7-ASK complex, and screening methods based on these methods. Moreover, the present invention relates to substrate compounds, antibodies or Cdc7-ASK complexes useful to these methods, or methods for preparing the compounds, antibodies or Cdc7-ASK complexes.

[1] A method for measuring the kinase activity of a Cdc7-ASK complex, comprising the following steps:

- (a) contacting a substrate protein with the Cdc7-ASK complex under conditions that allow phosphorylation of the substrate protein, wherein the substrate protein is a protein comprising the amino acid sequence of SEQ ID NO: 1, or a protein functionally equivalent to that protein;
- (b) measuring the level of phosphorylation of a serine residue of the substrate protein at the position corresponding to position 17 in the amino acid sequence of SEQ ID NO: 1; and
- (c) measuring the kinase activity of the Cdc7-ASK complex using the level of phosphorylation as an indicator.

[2] The method according to [1], wherein the level of phosphorylation is measured based on the level of binding of an antibody that identifies the level of phosphorylation of the serine residue.

[3] The method according to [1], wherein the Cdc7-ASK complex is derived from a biological sample.

[4] A method for measuring the effects of a test compound on the kinase activity of a Cdc7-ASK complex, comprising the following steps:

(a) contacting a test compound, a substrate protein and a Cdc7-ASK complex active substance, wherein the substrate protein is a protein comprising the amino acid sequence of SEQ ID NO: 1, or a protein functionally equivalent to that protein, and wherein they are contacted in any of the following orders i) to iii):

i) the test compound and substrate protein are contacted, followed by contacting the Cdc7-ASK complex active substance,

ii) the substrate protein and Cdc7-ASK complex active substance are contacted in the presence of the test compound, or

iii) the substrate protein and Cdc7-ASK complex active substance are contacted, followed by contacting the test compound;

(b) measuring the level of phosphorylation for a serine residue of the substrate protein at the position corresponding to position 17 in the amino acid sequence shown in SEQ ID NO: 1; and

(c) measuring the effect of the test compound on the kinase activity of the Cdc7-ASK complex active substance using the level of phosphorylation as an indicator.

[5] A method of screening for compounds comprising the effect of regulating the kinase activity of a Cdc7-ASK complex, comprising the following steps:

(a) measuring the effect of a test compound on the kinase activity of the Cdc7-ASK complex according to the method

described in [4]; and

(b) selecting a test compound with a high or low level of phosphorylation by comparison with a control that has not been contacted with the test compound.

[6] A screening method according to [5], wherein a compound having a low level of phosphorylation is selected in step (b) of [5].

[7] An inhibitor of cell growth comprising a compound selected according to the screening method of [6] as its active ingredient.

[8] A kit for measuring the kinase activity of a Cdc7-ASK complex comprising:

- (a) a substrate protein comprising a continuous amino acid sequence that comprises a serine residue at position 17 of the amino acid sequence of SEQ ID NO: 1, and that which is selected from the amino acid sequence of SEQ ID NO: 1; and
- (b) an antibody that identifies the level of phosphorylation of the serine residue of the substrate protein at the position corresponding to position 17 of the amino acid sequence of SEQ ID NO: 1.

[9] A kit for evaluating the effect of a test compound on the kinase activity of a Cdc7-ASK complex, comprising:

- (a) a Cdc7-ASK complex active substance; and,
- (b) a substrate protein comprising a continuous amino acid sequence that comprises the serine residue at position 17 of the amino acid sequence of SEQ ID NO: 1, and is selected from this amino acid sequence.

[10] A process for producing a Cdc7-ASK complex active substance comprising the following steps:

- (a) introducing a DNA encoding human Cdc7 protein and a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 10 or a protein functionally equivalent to the protein, into prokaryotic cells in a state that allows monocistronic expression;
- (b) expressing the two DNAs; and
- (c) recovering the expressed protein.

[11] An antibody that identifies the level of phosphorylation of the serine residue at position 17 of a protein comprising the amino acid sequence of SEQ ID NO: 1.

[12] A protein according to any of the following (a) to

- (d):
- (a) a protein comprising the amino acid sequence of SEQ ID NO: 1;

(b) a protein comprising a continuous amino acid sequence that is selected from the amino acid sequence of SEQ ID NO: 3, and comprises the serine of position 17;

(c) a protein comprising an amino acid sequence in which one or more amino acids in the amino acid sequence of SEQ ID NO: 1 are substituted, deleted, added and/or inserted, wherein the protein is phosphorylated by human Cdc7-ASK complex; and

(d) a protein comprising an amino acid sequence comprising 90% or more homology with the amino acid sequence of SEQ ID NO: 3, wherein the protein is phosphorylated by human Cdc7-ASK complex.

[13] A protein comprising a continuous amino acid that comprises the amino acid sequence of SEQ ID NO: 10 and that which is selected from the amino acid sequence of SEQ ID NO: 9.

[14] A polypeptide according to [13] that comprises the amino acid sequence of SEQ ID NO: 10.

Alternatively, the present invention relates to methods for inhibiting cell growth that comprise the step of administering a compound selected according to the screening method of [6]. In addition, the present invention relates to the use of a compound, selected according to the screening method of [6], in the production of a cell growth inhibitor.

The present invention relates to methods for measuring the kinase activity of Cdc7-ASK complex, where the methods comprise the following steps:

(a) contacting a substrate protein with the Cdc7-ASK complex under conditions that allow phosphorylation of the substrate protein, wherein the substrate protein is a protein comprising the amino acid sequence of SEQ ID NO: 1, or a protein functionally equivalent to that protein;

(b) measuring the level of phosphorylation of a serine residue of the substrate protein at the position corresponding to position 17 in the amino acid sequence of SEQ ID NO: 1; and

(c) measuring the kinase activity of the Cdc7-ASK complex using the level of phosphorylation as an indicator.

In the present invention, a protein comprising the amino acid sequence of SEQ ID NO: 1, or a protein functionally



equivalent to that protein, is used as a substrate protein.

The structure of MCM2 protein is known. The amino acid sequence of human MCM2, and the nucleotide sequence of the cDNA that encodes it, are shown in SEQ ID NOs: 2 and 3 respectively.

5 Cdc7-ASK complex phosphorylates a specific amino acid residue of the human MCM2 of SEQ ID NO: 3. The present inventors have previously demonstrated that Cdc7-ASK complex phosphorylates MCM complex or free MCM2. However, that the Cdc7-ASK complex specifically phosphorylates the serine at position 17 from the

10 MCM2 N-terminal is a novel finding, revealed by the present inventors.

Thus, proteins comprising continuous amino acid sequences that comprise the position 17 serine and are selected from the amino acid sequence of SEQ ID NO: 3, can be used as substrate

15 proteins in the present invention, as long as they can be phosphorylated by Cdc7-ASK complex. Examples of other substrate proteins that can be used in the present invention are indicated below. Tags can be added to these substrate proteins. In addition, these substrate proteins can be bound to a solid phase,

20 as described later.

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 1;
- (b) a protein comprising a continuous amino acid sequence that is selected from the amino acid sequence of SEQ ID NO: 3, and
- 25 comprises the serine of position 17;
- (c) a protein comprising an amino acid sequence in which one or more amino acids in the amino acid sequence of SEQ ID NO: 1 are substituted, deleted, added and/or inserted, wherein the protein is phosphorylated by human Cdc7-ASK complex; and
- 30 (d) a protein comprising an amino acid sequence comprising 90% or more homology with the amino acid sequence of SEQ ID NO: 3, wherein the protein is phosphorylated by human Cdc7-ASK complex.

In the present invention, the phosphorylation of a certain protein by the Cdc7-ASK complex can be confirmed based

35 on the results of, for example, the reactions described in Examples. Namely, a phosphate compound and the Cdc7-ASK complex

are incubated with a substrate protein under conditions that allow phosphorylation of that protein (described below). Next, the level of phosphorylation at the serine residue at the position corresponding to position 17 in the amino acid sequence shown in SEQ ID NO: 1 is measured. When there is no significant difference observed between the measured level of phosphorylation and the level when, for example, a protein comprising the amino acid sequence of SEQ ID NO: 1 is used as a substrate protein, then that protein is judged to be a protein phosphorylated by human Cdc7-ASK complex.

The number of amino acids that make up the substrate protein of the present invention can generally be five or more, usually ten or more, and preferably 50 or more. In addition, although there are no restrictions as to the size of the substrate protein, it can be a short fragment of, for example, 400, generally 300, or 200 or less. Phosphorylation by the Cdc7-ASK complex can be observed more specifically using a short fragment as a substrate protein.

In the present invention, proteins comprising, for example, the amino acid sequence of SEQ ID NO: 1, can be indicated as preferable substrate proteins. The amino acid sequence of SEQ ID NO: 1 is a fragment sequence comprising 130 amino acids on the N-terminal side of the human MCM2 of SEQ ID NO: 3. The serine located at the 17th amino acid counting from the N-terminal is phosphorylated by the Cdc7-ASK complex.

In addition, proteins which are phosphorylated by human Cdc7-ASK complex and that comprise amino acid sequences in which one or more amino acids in the amino acid sequence of SEQ ID NO: 1 are substituted, deleted, added and/or inserted, can also be used as substrate proteins. In the present invention the number of amino acids subjected to mutation is, for example, one to ten, preferably one to six, and more preferably one to three.

Mutation of an amino acid sequence may be a mutation that is induced artificially, or that occurs spontaneously. When substituting amino acids, conservative substitution can be used. In general, to maintain protein function, the substitute amino

acids are preferably those with properties similar to the amino acids prior to substitution. This type of amino acid substitution is referred to as conservative substitution.

For example, since Ala, Val, Leu, Ile, Pro, Met, Phe and Trp are all classified as non-polar amino acids, they have mutually similar properties. Examples of non-charged amino acids include Gly, Ser, Thr, Cys, Tyr, Asn and Gln. Alternatively, examples of acidic amino acids include Asp and Glu. Moreover, examples of basic amino acids include Lys, Arg and His. The amino acids that compose each of these groups have mutually similar properties. Consequently, there is a high possibility that protein function will be maintained when these amino acids are substituted with other amino acids from the same group.

This type of protein can be obtained by introducing a mutation into the nucleotide sequence of the cDNA of the human MCM2 of SEQ ID NO: 1. Technology for introducing mutations into genes comprising known nucleotide sequences is known. Alternatively, proteins comprising desired amino acid sequences can be prepared by chemical synthesis.

In the substrate proteins of the present invention, the serine located at the position corresponding to position 17 of the amino acid sequence of SEQ ID NO: 1 is important as the phosphorylation target of the Cdc7-ASK complex. Thus, even when the substrate protein is a protein comprising an amino acid sequence mutation, it is important that a serine be preserved at position 17, or a position homologous to position 17. The term "position homologous to position 17" refers to a position that corresponds to position 17 of the amino acid sequence of SEQ ID NO: 1, when a given amino acid sequence is aligned with the amino acid sequence of SEQ ID NO: 1. Multiple methods are known for aligning amino acid sequences. For example, a wide variety of software, based on algorithms such as the BLAST algorithm, is in practical use for aligning different amino acid sequences.

In addition, a protein that comprises an amino acid sequence comprising 90% or more homology to the amino acid

sequence of SEQ ID NO: 3, and which is phosphorylated by human Cdc7-ASK complex, can also be used as a substrate protein of the present invention. For example, human Cdc7-ASK complex phosphorylates not only human MCM2, but also mouse MCM2. Thus, mouse MCM2 can be used as a substrate protein in the present invention. The amino acid sequence of mouse MCM2 is shown in SEQ ID NO: 5, while the nucleotide sequence that encodes this amino acid sequence is shown in SEQ ID NO: 4. Mouse MCM2 and human MCMC2 are 99% homologous. Thus, proteins comprising 99% or more homology to the amino acid sequence of human SEQ ID NO: 3 (the full-length amino acid sequence of human MCM2) are useful as substrate proteins of the present invention.

In the present invention, the level of phosphorylation of the serine at position 17 of human MCM2, or of a serine at a position homologous to this serine, is measured as an indicator. When using a protein that comprises an amino acid sequence different from the amino acid sequences of SEQ ID NOs: 1 and 3 as a substrate protein, it is important to preserve the serine at position 17 of SEQ ID NO: 1, or serines at positions homologous to position 17. For example, in mouse MCM2, the serine at position 26 is the serine at a position homologous to position 17. Thus, when using mouse MCM2 fragments as substrate proteins, it is preferable to use proteins that comprise an amino acid sequence comprising a serine at position 26.

The substrate protein of the present invention can be obtained based on genetic engineering techniques. Namely, a protein having the desired amino acid sequence can be obtained by expressing a DNA encoding MCM2 in a suitable expression system. The nucleotide sequence of a DNA encoding MCM2 is shown in SEQ ID NO: 2.

For example, the Examples show an example of expressing the MCM2 gene using *Escherichia coli*. The expression product can be purified using techniques such as salting out, gel filtration, ion exchange chromatography or so on. Purification by affinity chromatography, using the binding affinity of a tag with binding affinity that has been fused to MCM2, can also be

used. Examples of binding tags that can be used include a histidine tag comprising several histidines (His-Tag),  $\beta$ -D-galactosidase, glutathione S-transferase (GST), thioredoxin, maltose-binding protein, Myc, Xpress, and FLAG. If GST is used, for example, the expressed protein can be purified easily with a Glutathione Sepharose 4B column or so on.

Depending on the expression system, MCM2 may be recovered in a phosphorylated state. When the substrate protein has been phosphorylated in the expression system, the protein can be dephosphorylated by treatment with an enzyme such as phosphatase, which acts on phosphate groups. The measurement methods of the present invention comprise the step of measurement using the phosphorylation of substrate proteins as an indicator. Thus, it is particularly preferable that the substrate protein sites to be phosphorylated by the Cdc7-ASK complex are not phosphorylated. In addition, methods are also known for chemically synthesizing proteins comprising the desired amino acid sequence.

The present invention's methods for measuring the kinase activity of Cdc7-ASK complex comprise the step of contacting the aforementioned substrate proteins with samples whose Cdc7-ASK complex kinase activity is to be measured, under conditions that allow phosphorylation of the substrate protein. In the present invention, "conditions that allow phosphorylation of the substrate protein" refer to conditions suited to expression of Cdc7-ASK complex kinase activity. More specifically, the temperature, salt concentration, and pH are suited to the expression of enzyme activity, and phosphate compounds are present for substrate protein phosphorylation. "Conditions suitable for enzyme activity" can be exemplified as below. Namely, such reaction conditions can be imparted by contacting the aforementioned components in HEPES buffer adjusted to pH 7.0 to 7.5.

In the present invention, "conditions that allow phosphorylation of a substrate protein" refer to contacting a sample with a substrate protein in the presence of a compound comprising a phosphate group necessary for substrate protein

phosphorylation. Phosphate groups can be supplied by, for example, coexisting with adenosine triphosphate (hereinafter described as ATP).

5 The substrate protein and phosphate compound that comprise the aforementioned reaction are preferably added in excess with regards to the Cdc7-ASK complex kinase activity in the sample. It is generally difficult to accurately predict the kinase activity of Cdc7-ASK complex contained in a sample. However, those with ordinary skill in the art are able to  
10 experimentally predetermine an adequate amount of substrate compound or phosphate compound, such that the components required by the reaction are sufficient for enzyme reaction. For example, when measuring a human-derived biological sample, the amount of substrate protein to be used is, for example, 0.1  
15  $\mu\text{g}$  or more, and generally 0.2  $\mu\text{g}$  to 1  $\mu\text{g}$ . The amount of phosphate compound to be added as ATP can be, for example, 0.01 mM or more, and generally 0.1 mM to 1 mM. Furthermore, phosphate compound is added in an amount that is necessary and sufficient for phosphorylation of the substrate protein used in  
20 the reaction system.

The reaction time for phosphorylation is typically ten minutes or more, and preferably 30 minutes or more. Reliable phosphorylation of substrate protein can be expected from, for example, 50 to 100 minutes of reaction. The time required for  
25 phosphorylation varies according to the amount of substrate protein used, and the level of Cdc7-ASK complex activity in the reaction solution. Those with ordinary skill in the art are able to suitably set the reaction time required for phosphorylation under given conditions.

30 In the measurement methods of the present invention, the level of substrate protein phosphorylation over a predetermined time relates to phosphorylation activity in the sample. Alternatively, the phosphorylation activity in a sample can also be measured by using, as an indicator, the reaction time  
35 required to phosphorylate a substrate protein to a predetermined level. Namely, the greater a sample's phosphorylation activity,

the faster the substrate protein phosphorylation will reach a predetermined level. Moreover, phosphorylation activity in the sample can also be measured by using the amount of sample required to phosphorylate the substrate protein to a predetermined level as an indicator. In this embodiment, the amount of sample required for phosphorylation is reversely proportional to the level of phosphorylation activity in the sample.

When the phosphorylation activity in a sample far exceeds that predicted, the majority of the substrate protein will be phosphorylated in a short period of time. If this happens, accurate evaluation of phosphorylation activity may be impossible. Thus, when such results are obtained, it is preferable to reduce the amount of sample added to the reaction, and remeasure phosphorylation activity.

The measurement methods of the present invention comprise the step of measuring the phosphorylation level of the substrate protein for the serine residue at the position corresponding to position 17 of the amino acid sequence of SEQ ID NO: 1. The level of phosphorylation at this position can be measured immunologically by using, for example, an antibody that recognizes the phosphorylated state of the serine residue at a position corresponding to position 17.

More specifically, an antibody can be used for which binding activity decreases with serine dephosphorylation, as compared to binding activity to an antigen-determining group comprising the phosphorylated serine residue of a protein with an amino acid sequence comprising a serine that corresponds to position 17. Conversely, antibodies that bind to antigen-determining groups comprising a non-phosphorylated serine residue, but for which bonding activity is decreased with phosphorylation of the serine residue, can also be used.

In the measurement methods of the present invention, antibodies that identify the level of phosphorylation of the aforementioned serine residue, used to measure the level of phosphorylation, can be obtained in, for example, the following

manner: First, a synthetic peptide that comprises a continuous amino acid sequence comprising the applicable serine residue can be used as the immunogen. The length of the synthetic peptide used as the immunogen can be at least three or more, for example  
5 five or more, normally ten or more, and preferably ten to 20 amino acids. A preferable example of an immunogen is an amino acid sequence composed of 15 continuous amino acids, containing serine at position 17, and selected from the amino acid sequence described in SEQ ID NO: 1. The position of the serine can be  
10 arbitrary. For example, in the Examples, a synthetic peptide was used as an immunogen that comprised an amino acid sequence in which serine was located in the center of 15 amino acids. The synthetic peptide can be bonded to a carrier protein. Keyhole Limpet hemocyanin and such can be used as the carrier  
15 protein.

The serine residue corresponding to position 17 in the immunogen can be phosphorylated in advance. Methods for obtaining synthetic peptides in which a specific amino acid has been phosphorylated are known. When the serine in the immunogen  
20 is phosphorylated, antibodies comprising the activity of binding to phosphorylated serine can be obtained.

Immunogens are administered to immunity animals by mixing with suitable adjuvants. Immunization is normally performed several times, and blood is collected from immunized animals  
25 after confirming an adequate rise in antibody titer. Serum recovered from the collected blood can be used as antiserum that contains an antibody of the present invention. Purified antibodies can be obtained by purifying immunoglobulins from such antisera.

30 Alternatively, cell lines that produce monoclonal antibodies can be established by immortalizing antibody-producing cells from the immunized animals, and then selecting clones that produce antibodies comprising the desired reactivity. For example, antibody-producing cells can be immortalized by  
35 fusion with cells such as myeloma cells, to produce hybridomas.

Antibodies can be confirmed to have desired reactivity by



using the synthetic peptide used as the immunogen. For example, antibodies that recognize phosphorylated substrate proteins can be obtained by selecting antibodies that bind to synthetic peptides comprising phosphorylated serine. Moreover, an antibody that specifically binds to a phosphorylated substrate protein can be obtained by removing from this antibody any antibodies that bind to synthetic peptides that comprise non-phosphorylated serine. Using this kind of selection process, antibodies that recognize the phosphorylated state of substrate proteins, essential to the present invention, can be obtained by treating with antiserum or purified antibody.

Hybridomas that produce monoclonal antibodies able to recognize the phosphorylated state of substrate proteins can be selected by screening antibodies produced by the hybridomas, according to similar selection processes.

In the measurement methods of the present invention, the aforementioned antibodies can be labeled in advance. Antibodies bound to phosphorylated sites can be easily detected by labeling antibodies. In addition, if the signals from labels can be amplified, measurements of higher sensitivity can be expected.

Arbitrary labeling components can be used for antibody labeling. For example, fluorescent dyes, enzymes, radioactive substances, and so on can be used as labels. In addition, substances with high electron density, such as ferritin and gold colloids, can also be used as labels.

Examples of fluorescent dyes include fluorescein isothiocyanate (FITC), rhodamine  $\beta$ -isothiocyanate (RITC), and phycoerythrin (PE). Examples of enzymes include peroxidase,  $\beta$ -D-galactosidase, microperoxidase, alkaline phosphatase, acidic phosphatase, and cytochrome c. Examples of radioactive substances include  $^{125}\text{I}$ ,  $^{14}\text{C}$  and  $^3\text{H}$ . Methods for labeling antibodies with these labeling components are known.

In addition, antibodies can also be labeled using antibodies (hereinafter referred to as secondary antibodies) that bind to phosphorylation-recognizing antibodies (primary antibodies). For example, a phosphorylation-recognizing

antibody is contacted with a substrate protein, and then reacted with a secondary antibody. The amount of secondary antibody indirectly bound to the substrate is measured, and the amount of bound primary antibody is then determined from the measured amount of secondary antibody. Thus, the level of substrate phosphorylation can be determined. For example, when using a mouse monoclonal antibody as a primary antibody, anti-mouse IgG goat antibody can be used as a secondary antibody.

Alternatively, a primary antibody can also be indirectly labeled by labeling with a substance with binding affinity, and then using the affinity of a binding partner to that affinitive substance. For example, if a primary antibody is avidinyllated, a biotinylated enzyme can be bound to the antibody using the affinity between avidin and biotin.

In the measurement methods of the present invention, Cdc7-ASK substrate protein can be used in an immobilized state by pre-binding it to an insoluble support. Immobilizing the substrate protein makes it easier to detect binding with the aforementioned phosphorylation-recognizing antibodies. In other words, bound and unbound antibodies are easily separated by contacting the antibodies with a substrate protein, removing the liquid phase, and washing the solid phase. The amount of antibody bound (or not bound) to the substrate protein can then be easily measured by measuring the antibody in the solid phase (or liquid phase). Immobilizing substrate proteins on a solid phase also contributes to their stabilization.

Insoluble supports used for immobilizing substrate proteins include resins such as polystyrene resins, polycarbonate resins, silicon resins and Nylon resins, glass, and micelle particles, and the material used is not particularly restricted. There are also no particular restrictions on the form of the insoluble support, and examples of forms that can be used include trays, spheres, rods, fibers, cells and test tubes. The substrate proteins can be immobilized on insoluble supports by physical adsorption or chemical bonding. The insoluble support on which a substrate protein has been immobilized can

also be blocked as necessary. Albumin, skim milk, and so on are used for blocking. Blocking the insoluble support inhibits non-specific antibody binding. In addition, blocking can also be expected to exhibit a protective effect on the substrate protein.

5 In the present invention, the amount of antibody bound (or not bound) to a substrate protein is correlated with a sample's Cdc7-ASK complex kinase activity. More specifically, when using, for example, an antibody that binds to an antigen-determining group comprising a substrate protein's  
10 phosphorylated serine, the amount of antibody bound to the substrate protein is proportional to the degree of kinase activity of the Cdc7-ASK complex in the sample. The kinase activity in a sample can also be quantitatively determined by comparison with the results of measurements obtained from a  
15 Cdc7-ASK complex standard, for which the level of kinase activity is already known.

The present invention measures the kinase activity of Cdc7-ASK based on the phosphorylation state of a substrate protein region phosphorylated by Cdc7-ASK. Thus, the kinase  
20 activity of Cdc7-ASK can be specifically measured, without being affected by other substances that comprise phosphorylation activity and which may coexist in the sample. As a result, the state of cell growth can be specifically detected by measuring Cdc7-ASK kinase activity based on the methods of the present  
25 invention.

Cdc7-ASK maintains a high level of activity, particularly in the S phase of the cell cycle, and its activity decreases in the G1 phase. Thus, measuring the activity of Cdc7-ASK kinase is useful as an indicator of a cell's transition from the G1  
30 phase to the S phase. The S phase is the period when cells proceed to replicate nucleic acids in preparation for cell growth. Thus, for example, finding a large number of cells in the S stage among the cells in a certain biological sample means that cell growth is progressing in that sample. More  
35 specifically, if a large number of cells with high-level Cdc7-ASK kinase activity are found in a cancer tissue by using a

measurement method of the present invention, this cancer tissue contains many actively growing cells. In other words, it is possible that the cancer is highly malignant.

In addition, the present invention relates to methods for measuring the effect of a test compound on the kinase activity of Cdc7-ASK complex, comprising the following steps:

(a) contacting a test compound, a substrate protein and a Cdc7-ASK complex active substance, wherein the substrate protein is a protein comprising the amino acid sequence of SEQ ID NO: 1, or a protein functionally equivalent to that protein, and wherein they are contacted in any of the following orders i) to iii):

i) the test compound and substrate protein are contacted, followed by contacting the Cdc7-ASK complex active substance,

ii) the substrate protein and Cdc7-ASK complex active substance are contacted in the presence of the test compound, or

iii) the substrate protein and Cdc7-ASK complex active substance are contacted, followed by contacting the test compound;

(b) measuring the level of phosphorylation for a serine residue of the substrate protein at the position corresponding to position 17 in the amino acid sequence shown in SEQ ID NO: 1; and

(c) measuring the effect of the test compound on the kinase activity of the Cdc7-ASK complex active substance using the level of phosphorylation as an indicator.

In the aforementioned method, the term "Cdc7-ASK complex active substance" refers to a complex that is functionally equivalent to the Cdc7-ASK complex. More specifically, a "Cdc7-ASK complex active substance" is defined as a complex comprising the function of phosphorylating a substrate protein serine that is to be phosphorylated. Thus, if it makes up a complex that comprises this activity, the complex can still be used as a Cdc7-ASK complex active substance of the present invention, even if the Cdc7 or ASK comprising the complex comprise structures different to that of human-derived proteins.

Whether a certain protein complex comprises the function

of phosphorylating a substrate protein serine to be phosphorylated can be confirmed based on, for example, a method such as that shown in the Examples. Namely, the aforementioned substrate protein and protein complex are incubated under  
5 conditions that allow phosphorylation of the substrate protein. If there is no significant difference observed when comparing the substrate protein phosphorylation level after this incubation with the level after incubation with human Cdc7-ASK complex, then that protein complex is judged to be functionally  
10 equivalent to Cdc7-ASK complex.

The structures of human-derived Cdc7 and ASK are known. The nucleotide sequence of a cDNA encoding human Cdc7 is shown in SEQ ID NO: 6, while an amino acid sequence of human Cdc7 is shown in SEQ ID NO: 7. In addition, the nucleotide sequence of  
15 a cDNA encoding human ASK is shown in SEQ ID NO: 8, while an amino acid sequence of human ASK is shown in SEQ ID NO: 9. With regards to these known Cdc7 and ASK, for example, proteins such as the following can be used for each subunit composing a Cdc7-ASK complex active substance of the present invention: In the  
20 present invention, those subunits that differ from naturally-occurring human Cdc7 or human ASK, but are capable of making up Cdc7-ASK complex active substances comprising kinase activity, are respectively referred to as Cdc7 subunits and ASK subunits.

First, proteins that can be used as the Cdc7 subunit are  
25 those that compose a complex comprising the effect of phosphorylating a substrate protein by forming a complex with an aforementioned human ASK, wherein they are encoded by the following polynucleotides:

- (a) a polynucleotide that comprises a region that encodes the  
30 nucleotide sequence of SEQ ID NO: 6;
- (b) a polynucleotide that comprises a nucleotide sequence that encodes the amino acid sequence of SEQ ID NO: 7;
- (c) a polynucleotide that encodes a protein that comprises an  
35 amino acid sequence in which one or more amino acids are substituted, deleted, added and/or inserted in the amino acid sequence of SEQ ID NO: 7, in which the polynucleotide composes a

complex having phosphorylation effect on a substrate protein by forming a complex with the aforementioned human ASK; and,

(d) a polynucleotide that hybridizes with a polynucleotide that contains a region that encodes the nucleotide sequence of  
5 SEQ ID NO: 6 under stringent conditions, in which the polynucleotide encodes a protein that composes a complex having phosphorylation effect on a substrate protein by forming a complex with the aforementioned human ASK.

In the present invention, a protein encoded by an  
10 aforementioned polynucleotide can be confirmed to make up a complex comprising the effect of phosphorylating a substrate protein by forming a complex with an aforementioned human ASK in the following manner: Namely, the aforementioned polynucleotide is first co-expressed in suitable host cells along with a DNA  
15 comprising a nucleotide sequence encoding human ASK. The phosphorylation activity of the resulting expression product is then evaluated by, for example, a method such as that described in the Examples, and then compared with Cdc7-ASK phosphorylation activity. As a result, if there is no significant difference  
20 observed between the two phosphorylation activities, the Cdc7 subunit making up the complex is judged to be a protein that comprises a complex comprising the effect of phosphorylating a substrate protein by forming a complex with the aforementioned human ASK.

25 On the other hand, proteins encoded by the following polynucleotides and comprising complexes that comprise the effect of phosphorylating substrate proteins by forming complexes with the aforementioned human Cdc7, can be used as ASK subunits that comprise Cdc7-ASK complex active substances of the  
30 present invention:

- (a) a polynucleotide that comprises a region that encodes the nucleotide sequence of SEQ ID NO: 8;
- (b) a polynucleotide that comprises a nucleotide sequence that encodes the amino acid sequence of SEQ ID NO: 9;
- 35 (c) a polynucleotide that encodes a protein that comprises an amino acid sequence in which one or more amino acids are

substituted, deleted, added and/or inserted in the amino acid sequence of SEQ ID NO: 9, in which the polynucleotide composes a complex having phosphorylation effect on a substrate protein by forming a complex with the aforementioned human Cdc7; and,

- 5 (d) a polynucleotide that hybridizes with a polynucleotide that comprises a region that encodes the nucleotide sequence of SEQ ID NO: 8 under stringent conditions, in which the polynucleotide encodes a protein that composes a complex having phosphorylation effect on a substrate protein by forming a  
10 complex with the aforementioned human Cdc7.

In the present invention, proteins encoded by the aforementioned polynucleotides can be confirmed to make up complexes comprising the effect of phosphorylating substrate proteins by forming complexes with the aforementioned human Cdc7  
15 in the following manner: Namely, an aforementioned polynucleotide is first co-expressed in suitable host cells along with a DNA comprising a nucleotide sequence that encodes human Cdc7. The phosphorylation activity of the resulting expression product is then evaluated by, for example, a method  
20 such as that described in the Examples, and then compared with Cdc7-ASK phosphorylation activity. As a result, if there is no significant difference observed between the phosphorylation activities of the two, the ASK subunit making up the complex is judged to be a protein that comprises a complex comprising the  
25 effect of phosphorylating a substrate protein by forming a complex with the aforementioned human Cdc7.

For example, the present inventors clearly demonstrated that a protein comprising an amino acid sequence corresponding to positions 174 to 349 from the N-terminal in the 674 amino  
30 acid sequence that comprises human ASK is a region essential to the formation of a complex with Cdc7 and for expression of kinase activity. An amino acid sequence that comprises this region is shown in SEQ ID NO: 10. Such human ASK protein fragments can be used as ASK subunits that compose the Cdc7-ASK  
35 complex active substance of the present invention, as long as they comprise the aforementioned activity.

In the present invention, polynucleotides that encode the aforementioned Cdc7 subunits or ASK subunits can be obtained by PCR or hybridized screening from an arbitrary cDNA library. Human libraries as well as those from mammals other than humans  
5 such as mice or rats, or eukaryotic cells such as *Caenorhabditis* and *Schizosaccharomyces*, can be used as the cDNA libraries.

The Cdc7-ASK complex active substances of the present invention can be extracted from cells or can be produced using genetic engineering techniques. Production methods using  
10 genetic engineering techniques are preferable since they allow a large amount of protein to be easily obtained. A complex comprising human Cdc7 and ASK expressed in insect cells, for example, can be used as a Cdc7-ASK complex active substance of the present invention.

For example, the present inventors have already succeeded in producing such complexes (J. Biol. Chem., Vol. 275, No. 37, 29042-29052, 2000). More specifically, the Cdc7-ASK complex active substances used in the present invention can be obtained by using insect cells in accordance with, for example, the  
15 following steps (a) to (c):

- (a) introducing a DNA encoding a human Cdc7 subunit and a DNA encoding human ASK subunit into insect cells;
- (b) co-expressing the aforementioned two types of introduced DNA in the aforementioned insect cells; and,
- 20 (c) purifying the expressed protein (complex).

Any polynucleotide described in the aforementioned (a) to (e) can be used for a DNA that encodes a human Cdc7 subunit used in the aforementioned step (a). Their forms are not particularly restricted, and include cDNA, genomic DNA and  
25 synthetic DNA. For example, DNAs comprising the nucleotide sequence shown in SEQ ID NO: 6 can be used as DNAs that encode a human Cdc7 subunit.

Similarly, any polynucleotide described in the aforementioned (a) to (e) can be used as a DNA that encodes a  
35 human ASK subunit used in the aforementioned step (a). For example, a DNA comprising the nucleotide sequence shown in SEQ



ID NO: 8 can be used as a DNA that encodes a human ASK protein.

Sf9 cells or Sf21 cells derived from *Spodoptera frugiperda*, or Tn5 cells derived from *Trichoplusiani*, can be used as the insect cells. These cells are also commercially available (Invitrogen or Pharmingen), and can also be obtained from ATCC. DNAs encoding a Cdc7 subunit and a ASK subunit can be introduced into insect cells using a baculovirus. For example, a recombinant virus is produced by homologous recombination by subcloning these DNAs into a transfer vector and simultaneously transfecting insect cells with the resulting plasmid and baculovirus DNA. This recombinant virus infects insect cells and co-expresses the Cdc7 subunit and ASK subunit within the insect cells. In order to increase the amount of protein expressed, the recombinant virus is preferably purified and amplified prior to expression of the protein.

Examples of transfer vectors that can be used include commercially available pVL1392 (Pharmingen), pPAK8 (Clontech), pAcUW51 (Pharmingen), pAcUW31 (Clontech), and pAcAB3 (Pharmingen). Transfer vectors capable of simultaneously subcloning a Cdc7 subunit and an ASK subunit are particularly preferable as transfer vectors. For example, two genes can be subcloned by using a transfer vector having two promoters, such as pAcUW51 (Pharmingen), or a transfer vector having three promoters, such as pAcAB3 (Pharmingen). Alternatively, different transfer vectors that respectively subclone the Cdc7 subunit and ASK subunit can also be used.

In addition, examples of baculovirus DNA that can be used include BaculoGold™ Linearized Baculovirus DNA (Pharmingen) and wild type Baculovirus AcNV DNA (Pharmingen, Invitrogen). Various types of kit are commercially available for expression systems using baculovirus, and any of these kits may be used.

The Cdc7 subunit and ASK subunit genes, which are exogenous genes, can be co-expressed in insect cells using the aforementioned transfer vectors and baculoviruses. The Cdc7-ASK complex active substances used in the present invention can be obtained by culturing transformed cells under conditions that

allow expression, and then recovering the expression product. To facilitate recovery, suitable tags can be fused to either Cdc7 or ASK or both.

5 A Cdc7- and ASK-subunit complex can be also obtained in prokaryotic cells, according to the methods described below. For example, when using *Escherichia coli* as the prokaryotic cells, a Cdc7-ASK kinase complex active substance can be prepared according to the following steps:

- 10 (A) introducing a DNA encoding human Cdc7 subunit and a DNA encoding a protein comprising the amino acid sequence described in SEQ ID NO: 10 or a protein functionally equivalent to the protein, into *Escherichia coli* in a state that allows monocistronic expression;
- (B) expressing the two DNAs; and
- 15 (C) recovering the expressed protein.

According to the present inventors' studies, in an *Escherichia coli* system, inserting and expressing a partial ASK (SEQ ID NO: 10), which is not the full length human ASK protein, eventually enabled acquisition of a Cdc7-ASK complex active  
20 substance that comprised kinase activity. In the present invention, a protein functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 10 refers to a protein that can form a complex with a Cdc7 subunit and express kinase activity.

25 Whether or not a certain protein expresses kinase activity by composing a complex with a Cdc7 subunit can be confirmed as follows: Namely, a DNA that encodes the protein is first co-expressed in suitable host cells along with a DNA comprising a nucleotide sequence that encodes human Cdc7. The  
30 phosphorylation activity of the resulting expression product is then evaluated by, for example, a method such as that described in the Examples, and this is then compared with the phosphorylation activity of Cdc7-ASK. As a result, if no significant difference is observed between the two  
35 phosphorylation activities, the ASK subunit that makes up the complex is judged to be a protein that makes up a complex

comprising the effect of phosphorylating a substrate protein by forming a complex with the aforementioned human Cdc7.

Proteins that retain the aforementioned activity and comprise homology of 90% or more with the amino acid sequence of SEQ ID NO: 10 can be indicated as functionally equivalent proteins. In addition, proteins comprising an amino acid sequence in which one or more amino acids are substituted, deleted, added and/or inserted in the amino acid sequence of SEQ ID NO: 10 that retain the aforementioned activity are included in the functionally equivalent proteins. The number of mutated amino acids is normally 20 or less, for example ten or less, and preferably five or less, for example, one to three. Amino acids can be substituted by conservative substitution. Proteins in which a tag has been fused to the amino acid sequence of SEQ ID NO: 10 are included in the functionally equivalent proteins of the present invention.

Step (A) uses a DNA encoding a human Cdc7 subunit similar to that previously described, and a DNA encoding a fragment sequence of a human ASK subunit (hereinafter abbreviated as "partial ASK"). A protein comprising the amino acid sequence shown in SEQ ID NO: 10, or a protein functionally equivalent to the protein, is used as the protein comprising the fragment sequence. The amino sequence of SEQ ID NO: 10 is a partial sequence corresponding to the amino acid sequence from the 174th to the 349th amino acids of SEQ ID NO: 9. The amino acid sequence described in SEQ ID NO: 11 is encoded by the nucleotide sequence described in SEQ ID NO: 10. The nucleotide sequence described in SEQ ID NO: 10 is a partial nucleotide sequence corresponding to the 1027th to the 1564th nucleotide of the nucleotide sequence of SEQ ID NO: 8. Protein fragments comprising this amino acid sequence are regions necessary for the expression of kinase activity by forming a complex with human Cdc7. Herein, as long as a partial ASK encodes a partial ASK protein, it is not limited to this sequence, and DNAs comprising an arbitrary nucleotide sequence that takes codon degeneracy into account are included. The form of this DNA is

also unrestricted, and includes cDNA, genomic DNA and synthetic DNA.

In the present invention, the term "a state that allows monocistronic expression" refers to transcribing a DNA encoding  
5 a Cdc7 subunit and a DNA encoding an ASK subunit as a single mRNA molecule, and translating them in to two protein molecules. In order to allow monocistronic expression, these DNAs are arranged so as to enable expression under the regulation of a common transcription regulatory region. They are designed so  
10 that a nucleotide sequence such as a terminator, which terminates transcription, is not contained between the two genes. The two genes are preferably arranged in close proximity. However, if the two translation frames are consecutive, the genes will be translated as a single fusion protein, and thus a  
15 stop codon is arranged between the two genes.

In addition, arranging a ribosome binding sequence (RBS; Shine-Dalgarno sequence) between the two genes is effective in facilitating efficient translation of each of the two mRNA protein-encoding regions. In the Examples, a construct is  
20 indicated for monocistronic expression in *Escherichia coli* of human Cdc7 and human partial ASK protein comprising the amino acid sequence of SEQ ID NO: 10. The vectors used to produce the construct are not limited. More specifically, by sequentially cloning, for example, DNAs encoding a Cdc7 subunit and an ASK  
25 subunit into the cloning site of a commercially available vector such as pGEX-2T, as indicated in the Examples, a vector that allows their monocistronic expression can be obtained.

A desired Cdc7-ASK complex active substance can be obtained by transforming this vector construct in *Escherichia coli* and recovering the expression product from the culture,  
30 according to established methods.

The expression product can be purified by a technique such as salting out, gel filtration, or ion exchange chromatography. In methods for producing a complex that use  
35 these genetic engineering techniques, a tag can be fused to either or both of the subunits that comprise the complex.

Purification can also be used in which a tag with binding affinity for the subunits is fused, followed by affinity chromatography using a substance with binding affinity for this tag. Examples of binding tags that can be used include  
5 histidine tags comprising several histidines (His-Tag),  $\beta$ -D-galactosidase, glutathione S-transferase (GST), thioredoxin, maltose-binding protein, Myc, Xpress, and FLAG. If GST is used, for example, the expressed protein can be purified easily with a Glutathione Sepharose 4B column or such.

10 A substrate protein for measurement of kinase activity according to the present invention can be used as a substrate protein for use in the measurement methods of the present invention, as previously described. In the present invention, a test compound is contacted with a substrate protein and a Cdc7-  
15 ASK complex active substance in any of the orders described in the aforementioned i) to iii).

i) By contacting a test compound with a substrate protein, and then contacting the Cdc7-ASK complex active substance, a kinase-modifying effect can be found in the action of the test  
20 compound on the substrate protein. ii) By contacting a substrate protein with a Cdc7-ASK complex active substance in the presence of a test compound, competitive inhibitory activity on the kinase activity of the Cdc7-ASK complex active substance can be evaluated. Furthermore, iii) By contacting a substrate protein  
25 and Cdc7-ASK complex active substance, then contacting a test compound, the dephosphorylation effect of the test compound on the phosphorylated substrate protein can be detected.

In the present invention, the level of phosphorylation of a substrate protein can be measured according to methods similar  
30 to methods for measuring the kinase activity of the present invention, as previously described. For example, the level of phosphorylation is measured by an antibody that recognizes the phosphorylated state at a phosphorylated site of a substrate protein.

35 The results of measuring the phosphorylation level are correlated with a test compound's effect on the kinase activity

of the Cdc7-ASK complex active substance. For example, when the phosphorylation level is reduced in comparison with a control that has not been contacted with a test compound, the test compound is concluded to comprise the activity of inhibiting phosphorylation. In addition, when the level of phosphorylation increases due to a test compound, the test compound is concluded to comprise the effect of promoting the kinase activity of a Cdc7-ASK complex active substance.

Phosphorylation levels can be compared with a control that has not been contacted with a test compound, as well as with the measurement results of a compound that clearly acts on kinase activity. For example, to discover inhibitory effect on kinase activity, the level of a test compound's inhibitory effect can be also evaluated by comparison with a compound already confirmed to comprise a certain degree of inhibitory effect. Furthermore, effects can be evaluated quantitatively by performing an aforementioned measurement method on a substance whose inhibitory effect on kinase activity has already been determined, and then comparing these results with the measurement results of a test compound.

Screening methods for compounds comprising the effect of regulating Cdc7-ASK complex kinase activity can be performed based on methods for measuring the effect of a test compound on Cdc7-ASK complex kinase activity, according to the present invention. Namely, the present invention relates to methods of screening for compounds comprising the effect of regulating Cdc7-ASK complex kinase activity, comprising the following steps:

- (a) measuring the effect of test compounds on Cdc7-ASK complex kinase activity using an aforementioned method; and
- (b) selecting test compounds that have a high or low level of phosphorylation on comparison with a control.

In the screening methods of the present invention, phosphorylation level can not only be compared with a control that has not been contacted with a test compound, but also with the measurement results of a compound clearly demonstrating an

effect on kinase activity. For example, to discover a kinase activity inhibitory effect, the level of a test compound's inhibitory effect can also be evaluated by comparison with a compound already confirmed to comprise a certain degree of inhibitory effect. Compounds comprising an inhibitory effect above a constant level can then be screened by selecting compounds comprising a comparatively higher level of inhibitory effect.

In the screening methods of the present invention, examples of substances that can be used as test compounds include natural or synthetic proteins, peptides, antibodies, cell extracts from animals, plants or bacteria, culture supernatants, and low molecular weight compounds. These test compounds can be obtained from compound libraries or gene libraries.

When the test compound is a natural ingredient, a single compound that inhibits kinase activity can ultimately be identified by fractionating each test compound according to methods known to those skilled in the art (for example, various types of chromatography), and then detecting those test compounds. Compounds inhibiting or promoting kinase activity and isolated by the screening can be used as agents for regulating Cdc7-ASK complex kinase activity.

Cdc7-ASK complex serves as a key cell growth factor in the living body. Thus, cell growth can be regulated by regulating Cdc7-ASK complex kinase activity. For example, compounds that inhibit Cdc7-ASK complex kinase activity are useful as cell growth inhibitors. More specifically, compounds comprising Cdc7-ASK complex inhibitory effect, and selected according to the screening methods of the present invention, are useful for regulating cells such as cancer cells, whose growth should be inhibited.

The screening methods of the present invention specifically detect phosphorylation of a substrate protein by Cdc7-ASK complex. Thus, the effect on Cdc7-ASK complex of compounds selected by the screening methods of the present

invention can be said to be more specific. When using such compounds to regulate cancer, for example, the resulting agents can be expected to be highly selective for proliferating cells, since they act specifically on cells in the growth phase.

5        These compounds are particularly useful as candidate compounds for cancer therapeutic agents. When using a compound isolated according to the screening methods of the present invention as an agent to adjust kinase activity, that compound can be prepared for use in accordance with known pharmaceutical  
10        production methods. For example, the compound can be administered to a patient along with a pharmaceutically acceptable carrier or medium (such as physiological saline, a vegetable oil, suspending agent, surfactant or stabilizer). Administration may be performed transcutaneously, nasally,  
15        transtracheally, intramuscularly, intravenously, or orally, according to the properties of the compound. Although the dosage varies according to patient age and body weight, patient condition, administration method, and so on, those with ordinary skill in the art can select a suitable dosage.

20        In addition, the present invention relates to a kit comprising an antibody capable of recognizing the phosphorylated state of a substrate used in the aforementioned measurement methods or screenings. When used to detect kinase activity, kits of the present invention comprise, for example, substrate  
25        proteins, buffers and so on, in addition to the aforementioned antibody. In addition, when used to screen for compounds that inhibit or promote kinase activity, Cdc7-ASK complex active substance is also included. Either the substrate protein or the antibody can be labeled as previously described, while the other  
30        can be immobilized.

      An enzyme standard and substrate protein standard can be included in the kit in order to test the activity of the Cdc7-ASK complex active substance and the measuring system itself. Other ingredients can be added to these standards and above-  
35        mentioned antibodies for the purpose of stabilization or such. For example, BSA at about 1%, or polyoles such as sucrose and



fructose at a final concentration of 0.2% to 10% (preferably 1%), can be added to the standards as protein denaturation preventives after freeze-drying.

All prior art references cited herein are incorporated by  
5 reference in the present specification.

#### Brief Description of the Drawings

Fig. 1 shows the arrangement of motif-M and motif-C in human ASK, budding yeast Dbf4, and fission yeast Him1/Cfpl.

10 Fig. 2 shows results that confirm the specificity of anti-Mcm2-phospho-S17 polyclonal antibody. Absorbance at 450 nm is plotted on the vertical axis, while antibody concentration (ng/ml) is plotted on the horizontal axis.

15 Fig. 3 shows the results of measuring the protein phosphorylation activity of Cdc7-ASK complex active substance. Absorbance at 450 nm is plotted on the vertical axis, and the amount of enzyme ( $\mu$ L) is plotted on the horizontal axis.

20 Fig. 4 shows the results of investigating changes in reactivity when the concentration of ATP added to a phosphorylation reaction solution was changed, in a method for measuring Cdc7-ASK complex activity. The relative activity value (%) based on a reactivity of 100 at 2 mM, is plotted on the vertical axis. The final ATP concentration (mM) in the reaction solution is plotted on the horizontal axis.

25 Fig. 5 shows the results of investigating changes in reactivity when the phosphorylation reaction time was changed, in a method for measuring the activity of Cdc7-ASK complex. Absorbance at 450 nm is plotted on the vertical axis, while the reaction time (minutes) is plotted on the horizontal axis.

30 Fig. 6 shows the results of evaluating a method for measuring phosphorylation activity by ELISA using Cdc7-ASK complex (wild type, WT) and inactive Cdc7-ASK complex (KD). Absorbance at 450 nm is plotted on the vertical axis, while the amount of enzyme ( $\mu$ L) is plotted on the horizontal axis.

35 Fig. 7 shows the results of measuring phosphorylation activity by radiofilter assay and ELISA method. The ELISA

results (absorbance at 450 nm) are plotted on the left vertical axis; the radiofilter assay results (radioactivity; cpm) are plotted on the right vertical axis; and the amount of enzyme ( $\mu$ L) is plotted on the horizontal axis.

Fig. 8 shows the results of measuring the effects of known protein phosphorylation inhibitors on Cdc7-ASK complex phosphorylation activity. Relative inhibition value (%) is plotted on the vertical axis, while inhibitor concentration ( $\mu$ M) is plotted on the horizontal axis.

#### Best Mode for Carrying Out The Invention

The following provide a more detailed explanation of the present invention based on Examples.

All commonly used techniques described in the Examples were carried out in accordance with the publications below. The methods introduced in the various publications can be used for the experimental methods used in the Examples, without any particular restriction.

J. Sambrook, E. F., Fritsch & T. Maniatis (1989), "Molecular Cloning, a laboratory manual, second edition", Cold Spring Harbor Laboratory Press;

Ed Harlow and David Lane (1988), "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory Press;

S. Oumi, K. Tsujimura & M. Inagaki (1994), "Cellular Engineering Supplement, Experimental Protocol Series, Anti-peptide antibody experimental protocols", Shujunsha Co., Ltd.; and

T. Migita, S. Konda, H. Motomoro & T. Hamaoka (1995), "Immunology Experimental Procedures I and II", Nankodo Co., Ltd.

#### Example 1. Identification of ASK Activity Domain

The present inventors conducted detailed mutation analyses using fission yeast Hsk1-Him1/Dfp1 as a material. As a result, the present inventors reported that the presence of only Dbf4-motif-m and Dbf4-motif-C is sufficient for kinase activation (Ogino, K. et al., J. Biol. Chem. 276: 31376-31387, 2001).

Fission yeast Hsk1-Him1/Dfp1 is a complex that corresponds to human Cdc7-Dbf4. On the basis of this fact, the present inventors predicted that a minimum domain comprising only motif-M and motif-C would be also be adequate for kinase activation in human ASK. Fig. 1 shows the results of comparing the arrangement of motif-M and motif-C in human ASK, budding yeast Dbf4, and fission yeast Him1/Cfp1.

More specifically, an amino acid sequence corresponding to the 173rd to 349th residue (SEQ ID NO: 10) was selected from the amino acid sequence of human ASK, shown in SEQ ID NO: 9. The DNA encoding this region is precisely divided at introns. The region was selected because introns are advocated to frequently exist at the divisions between functional domains or functional modules.

#### Example 2: Vector for Expression of Cdc7-ASK Complex Active Substance

A plasmid designated as GST-TAT-ASK(minimum)-HA-huCdc7 was prepared in order to express the active domains of human Cdc7 and ASK in *E. coli*. First, a GST-TAT-ASK-fused protein expression vector, GST-TAT11, was prepared. A restriction enzyme site (NotI) was then inserted downstream of ASK, and HA-Cdc7 was inserted at this site. At this time, a ribosome binding sequence (RBS; Shine-Dalgarno sequence) was added immediately in front of the HA-Cdc7. Due to the addition of this potent RBS, the section from GST-TAT-ASK to HA-Cdc7 was transcribed as one sequence, and both proteins could be efficiently translated from a single transcription product. This detailed procedure is described below:

A region that encodes from the 173rd to the 349th amino acid of the cDNA of human ASK was amplified by PCR using the two primers below:

Minimum ASK-N (HindIII): CCC AAG CTT GAC ATT AGA TAC TAC ATT GAA (SEQ ID NO: 11); and

Minimum ASK-C (EcoRI): CCG GAA TTC TTT CTT TTT AGG TGT GTC CTT (SEQ ID NO: 12).

The resulting amplification products were purified, and then cloned into a GST-TAT11 vector. Each region is arranged in this vector in the order of GST-TAT-HindIII-ASK-EcoRI. pGEX-2T-Tat11-TK vector was used as the GST-TAT11 vector. This vector  
 5 comprises a structure in which a TAT sequence comprising 11 amino acids (YGRKKRRQRRR/SEQ ID NO: 13) is inserted into a GST vector. The HindIII-EcoRI site of TK can be digested to insert minimum ASK, which has been separately amplified with PCR. Since the HindIII frame is AAG CTT, translation in this vector  
 10 terminates immediately on reaching the stop codon in the 3FRAME, just downstream of the EcoRI site.

Next, the EcoRI site of GST-TAT-HindIII-ASK-EcoRI was changed to a NotI site using an EcoRI-NotI adapter (AATTGCGGCCGC/SEQ ID NO: 14). The full-length coding region was  
 15 then amplified from the cDNA of human Cdc7, using the primers below:

huCdc7-RBS-N(NotI)/: A TAA GAA TGC GGC CGC TAA Gaa gga gAT  
 ATA CAT atg TAC CCC TAC GAC GTG (SEQ ID NO: 15); and

huCdc7C-NotI: ATAAGAATGCGGCCGCTTATCACAAGCTCATATCTTT (SEQ ID  
 20 NO: 16).

In the nucleotide sequence of huCdc7-RBS-N(NotI), regions corresponding to RBS and ATG are indicated by lower case letters. The amino acid sequence encoded by the nucleotide sequence starting at ATG is MYPYDVPDYAFSPQRD (SEQ ID NO: 17). The  
 25 MYPYDVPD of this sequence corresponds to the HA epitope. With this vector, a protein is expressed that comprises an amino acid sequence in which the amino acid sequence starting at the 14th amino acid of human Cdc7 is coupled with the HA tag. As previously mentioned, both genes are translated due to the  
 30 addition of potent RBS.

Furthermore, wild type and kinase-deactivated type Cdc7 were prepared. The kinase-deactivated type is referred to as K90E, which comprises an amino acid sequence in which the 90th amino acid, lysine (K), is mutated to glutamic acid (E).

### Example 3: Expression and Purification of Cdc7-ASK Complex

### Active Substance in Bacteria

*E. coli* C600lon- was transformed with the vector GST-TAT-ASK(minimum)-HA-huCdc7, constructed in Example 2 above, in accordance with ordinary methods. C600lon- is a strain of *E. coli* that is missing lon, which is a major *E. coli* protease.

The transformed *E. coli* was inoculated into 200 ml of LB medium containing 40 µg/ml of ampicillin, and cultured at 37°C. When the OD<sup>600</sup> of the culture medium reached 0.5, IPTG was added at a concentration of 1 mM, and then cultured for an additional three hours. The bacteria were collected by centrifugation, washed, and then suspended in 20 ml of buffer A (40 mM Hepes/KOH (pH 7.6), 1 mM EDTA, 40 mM potassium glutamate, 10% glycerol, and 1 mM DTT). The cells were disrupted using ultrasonic treatment, and the resulting products were fractionated into a soluble fraction and a precipitate (insoluble fraction) by centrifugation. 1 ml of glutathione Sepharose 4B was added to the soluble fraction. After stirring at 4°C for one hour, the glutathione Sepharose 4B was recovered by centrifugation, packed into a simple column, and washed well with buffer A. The column was washed until the OD<sup>280</sup> of the eluate reached 0.01 or less, using an amount of buffer A equal to or more than 20 times the column volume. Subsequently, the glutathione Sepharose 4B was eluted first with buffer A containing 20 mM glutathione, and then with buffer A containing 50 mM glutathione.

The fraction finally eluted was separated by SDS-PAGE, and then silver stained, confirming the purification of GST-ASK (about 50 kDa) and HA-Cdc7 (about 65 kDa).

### Example 4: Expression and Purification of MCM2(1-130) by Bacteria

Total RNAs were prepared from Jurkat cells using ISOGEN (Nippon Gene Cat. No. 311-02501), and cDNA was produced using You-Prime First-Strand Beads (Amersham-Pharmacia Cat. No. 27-9264-01). Using this cDNA as a template, human Mcm2(1-130) cDNA was then amplified using the Expand High Fidelity PCR System (Boehringer-Mannheim Cat. No. 1732-650) and the primers below.

The resulting 390 bp PCR product was digested with the BamHI and XhoI restriction enzymes, and then cloned into the BamHI/XhoI site of the *E. coli* expression vector, pGEX-4T-1 (Amersham-Pharmacia). The nucleotide sequence was determined, and the sequence of the resulting cDNA was confirmed to be human MCM2(1-130). The primers used for PCR are shown below:

Forward: 5'-CAC GGA TCC ATG GCA TCC AGC CCG GCC CA-3' (SEQ ID NO: 18); and

Reverse: 5'-GTG CTC GAG CAT CGC TGT CAT ACA GGA GCC-3' (SEQ ID NO.: 19).

*E. coli* DH5a was transformed with MCM2(1-130)/pGEX-4T-1. This *E. coli* was cultured for about five hours at 30°C, and the culturing temperature was lowered to 20°C when the optical absorbance (600 nm) reached 0.8. IPTG was added to a final concentration of 0.2 mM, and culture was continued for a further 16 hours at 20°C. The collected *E. coli* were suspended in ice-cold solubilizing buffer (PBS, 0.1% TritonX-100 and 1 mM PMSF) and dissolved by disrupting with an ultrasonic crusher on ice. The dissolved crude extract was centrifuged for 30 minutes at 15,000 rpm with a high-speed centrifuge to separate the soluble and insoluble fractions. Each fraction was subjected to SDS-PAGE, and the GST-fusion Mcm2(1-130) was confirmed to be in the soluble fraction. The soluble fraction was added to a column filled with 2 ml of GSH-Sepharose 4B (Amersham-Pharmacia). The column was then washed with 50 ml of washing solution (20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 5 mM imidazole), and then eluate (10 mM glutathione and 50 mM Tris-HCl (pH 9.6) was used to elute 2 ml into each of ten containers. Optical absorbance (A280 nm) was measured, and that with absorbance of 0.1 or more was dialyzed overnight against ice-cold PBS. Western Blotting using anti-MCM2-specific antibody confirmed that the dialyzed protein was GST-fusion MCM2(1-130).

#### Example 5: Identification of Phosphorylated Site of MCM2

First, the primary structures of human, mouse, frog, drosophila, fission yeast, budding yeast, and so on were aligned

to identify preserved serine and threonine residues. This was based on the hypothesis that functionally important phosphorylation sites are preserved across species. Eight sites (F1, F2, F3, F4, F5, F6, NF1 and NF2) were selected where these  
5 preserved serine and threonine residues formed clusters, being present in relatively large numbers.

Variant MCM2, in which serine and threonine residues 4 to 7 were substituted with alanine or glutamic acid at each site, was prepared for each region. Variants were prepared according to  
10 methods that use oligonucleotides. They were then cloned into insect cell expression vector, UW31. UW31 is a vector that can simultaneously express two types of gene products. Recombinant virus solutions co-expressing histidine-tagged MCM7 along with each variant MCM2 were obtained. These were then co-infected  
15 into Sf9 insect cells together with a recombinant virus solution that co-expresses histidine-tagged MCM4 and MCM6, and cell extracts were prepared. Affinity purification was then performed on the MCM-2-4-6-7 complex using a nickel column.

The fractions into which the desired MCM-2-4-6-7 complex eluted were pooled. Next, the MCM-2-4-6-7 complex was further  
20 purified using a mono-Q column (using the Pharmacia SMART System). The peak fractions eluting at 0.3 to 0.35 M NaCl were pooled. After dialyzing to a lower salt concentration, the fraction was used as a substrate for *in vitro* phosphorylation  
25 reactions.

In addition, and at the same time, polypeptides comprising a portion of these preserved sites were prepared with a histidine or GST tag, expressed in *E. coli*, and then purified. They were prepared by amplifying the intended coding region  
30 using a primer oligonucleotide to which a restriction enzyme site had been added, then cloning into a histidine-tagged expression vector (pT7-7/pQE30) or GST-fusion expression vector (pGEX-5X-3). The wild type and an alanine-substituted variant were expressed. These expression vectors were respectively  
35 inserted into strain DE3 or C600lon-, and their expression was induced. Affinity purification was then carried out with a

nickel column or glutathione Sepharose bead column. These proteins were also used as substrates for *in vitro* phosphorylation reactions.

When the substrate was MCM2-4-6-7 protein complex  
5 comprising variant MCM2, phosphorylation was not completely absent regardless of the variant used. Therefore, the phosphorylated wild type and variant MCM2 were digested with trypsin, and then spread out using two-dimensional chromatography to analyze whether or not the spots had  
10 disappeared and the phosphorylation sites were restricted.

In addition, as a result of phosphorylation by Cdc7, the SDS-PAGE mobility of MCM2, both *in vivo* and *in vitro*, shifts downward. Using this phenomenon as an indicator, an attempt was made to identify variants without the shift.

15 From these results, sites phosphorylated by the Cdk and Cdc7 of the MCM2 N-terminal (S27/S41 and S26/S40) were identified. Moreover, this phosphorylation was clearly a cause of the SDS-PAGE shift. The serine residue at position 17 of human MCM2 (S17), which corresponds to the serine residue at  
20 position 26 of mouse MCM2 (S26), was demonstrated to be efficiently phosphorylated by Cdc7 when a polypeptide of 130 amino acids from the N-terminal was used as a substrate. On the basis of these results, a protein comprising 130 amino acids from the N-terminal of human MCM2 (SEQ ID NO: 1) was used as a  
25 substrate protein for an ELISA assay, described below.

#### Example 6: Production of Anti-Phosphorylated Peptide Antibody

Since it is clear that the Cdc7-ASK complex specifically phosphorylates the 17th serine residue of human MCM2, an anti-  
30 phosphorylation-specific antibody that specifically recognizes the phosphorylated 17th serine residue was next produced, as a tool for detecting phosphorylation of the 17th serine residue.

The following two peptides were produced using a peptide synthesizer, and correspond to the amino acid sequence from the  
35 10th to the 20th residue of the human MCM2 protein, comprising the 17th serine residue found to be the phosphorylated site:



Phosphorylated peptide (written as Mcm2-phospho-S17):  
CRGNDPLTS(p)S (SEQ ID NO: 20); and

Non-phosphorylated peptide (written as Mcm2-S17): CRGNDPLTSS  
(SEQ ID NO: 21).

5       The aforementioned peptide sequences are depicted using the  
single-letter code in the direction from the amino terminal to  
the carboxyl terminal. "S(p)" indicates the phosphorylated  
serine residue. The cysteine (C) residue of the amino terminal  
was introduced to allow the peptide to covalently bond with a  
10       carrier protein. Using HPLC, these peptides were confirmed to  
have a purity of 95% or more. Phosphorylated peptides were used  
as immunogens. Phosphorylated peptides and non-phosphorylated  
peptides were used for measuring antibody titer and purifying  
antibodies.

15       Alone, the synthesized short peptide has low antigenicity.  
Therefore, animals are generally immunized using peptides that  
have been bound to carrier proteins. Albumin, myoglobin,  
hemocyanin and so on can be used as carrier proteins, however,  
hemocyanin (keyhole limpet hemocyanin, hereinafter KLH;  
20       CALBIOCHEM) was used this time. The carrier protein was  
covalently bonded using m-maleimidobenzoyl-N-hydroxysuccinimide  
ester (MBS; SIGMA), which is an agent that crosslinks phospho-  
Hs-S83 and KLH, in accordance with the attached protocol, and  
KLH-Mcm2-phospho-S17 was synthesized.

25       20 µg/100 µl and 10 µg/100 µl of KLH-Mcm2-phospho-S17 were  
used to respectively immunize rabbits and mice once. 100 µl of  
Freund's complete adjuvant (Yatron) was then added. Immunogens  
were then prepared by emulsification. The rabbits were  
immunized by subcutaneous injection to the back. Immunization  
30       was carried out from three to five times every two weeks. After  
blood was collected from the auricular vein, serum was recovered.  
Antibody titer was measured by ELISA using a microtiter plate on  
which Mcm2-phospho-S17 and Mcm2-S17 were immobilized.

35       Adequate antibody titer was confirmed, and from the next  
week, a cycle consisting of blood collection (during the first  
week), rest (during the next week) and immunization (during the

final week) was repeated four times. Blood was collected from the auricular vein in the same manner as for titer confirmation. Approximately 60 to 70 ml of blood was collected each time. In the final blood collection, blood was collected directly from the heart using a catheter.

The collected blood was allowed to stand overnight at 4°C, serum was separated from clot, and the serum in the supernatant was recovered. Ammonium sulfate at a final concentration of 50% was added to the separated and recovered serum. The mixture was stirred, and then separated by centrifugation. The minimum amount of PBS was added to the precipitate containing the IgG fraction, completely dissolving it. The solution was then dialyzed against PBS. After completely equilibrating with the PBS, the partially purified antibody fraction was applied to a column, and the antibody was purified.

Mcm2-phospho-S17 synthetic peptide was used to produce the specification column, while Mcm2-S17 synthetic peptide was used to produce the absorption column. 1 mg of synthetic peptide was dissolved in 5 ml of 0.1 M carbonate buffer, and then added to CNBr-activated Sepharose 4B (Pharmacia) equilibrated with 1 mM hydrochloric acid. This was gently mixed overnight at 4°C, and added to a column. The column was washed with 5- to 10-fold PBS, and equilibrated with 1 M Tris-HCl (pH 7.5), to block the active groups remaining on the surface of the Sepharose 4B. After blocking, the column was washed and equilibrated with PBS, and used for antibody purification.

To purify the anti-phosphorylated peptide antibodies, the aforementioned partially purified antibody fraction was passed through a specification column. After washing with PBS/0.1% TritonX-100, the anti-phosphorylated peptide antibodies adhered to the column were eluted with 0.17 M glycine-HCl (pH 2.5). The eluted antibodies were immediately neutralized by addition of a suitable amount of 1 M Tris-HCl (pH 8.0), and dialyzed against PBS to obtain anti-Mcm2-phospho-S17 antibody fraction. This was completely equilibrated with PBS, and then passed through the Mcm2-S17 Sepharose 4B column, an absorption column. Passing

through this absorption column removes antibodies that also react with the non-phosphorylated peptides present in the anti-Mcm2-phospho-S17 antibody fraction. Anti-phosphorylated peptide-specific antibodies pass through the column without  
5 being adsorbed. The absorption column was washed with PBS/0.1% TritonX-100. Antibodies bound to the absorption column were eluted with 0.17 M glycine-HCl (pH 2.5). Following elution, the column was again equilibrated with PBS/0.1% TritonX-100, and the anti-Mcm2-phospho-S17 antibody fraction was repeatedly passed  
10 through the absorption column until the non-phosphorylated peptide antibodies were almost completely absorbed. ELISA using a non-phosphorylated peptide-sensitized plate was used to confirm that the antibodies that react with non-phosphorylated peptides were removed. Specificity to the phosphorylated  
15 peptide was ultimately confirmed by ELISA using a phosphorylated peptide-sensitized plate.

The peptide was dissolved in 0.1 M carbonate buffer at a concentration of 1  $\mu\text{g/ml}$ . This was dispensed at 50  $\mu\text{l/well}$  to each well of a 96-well microtiter plate for ELISA. This was  
20 then left overnight at 4°C to sensitize. Following sensitization, the peptide solution was removed, a blocking solution (1% BSA, 5% sucrose and 0.1%  $\text{NaN}_3/\text{PBS}$ ) was added to each well at 200  $\mu\text{l/well}$ , and the plate was allowed to stand for about one hour at room temperature. The blocking solution was  
25 then completely removed, followed by air-drying in a draft chamber (immobilization).

The plates on which phosphorylated peptide (Mcm2-phospho-S17) were immobilized were used to measure each antibody titer, confirm absorption of anti-phosphorylated-serine-residue-specific antibody, and confirm the specificity of the anti-phosphorylated-peptide antibody. In addition, plates on which  
30 non-phosphorylated peptide (Mcm2-S17) was immobilized were used to confirm column absorption of non-specific antibodies.

The serum for testing antibody titer was serially diluted  
35 in 4-fold increments, starting from a 200-fold dilution using PBS. Purified antibody was serially diluted in 4-fold

increments starting from a concentration of 1  $\mu\text{g/ml}$  using PBS. The diluted samples were added to the sensitized plates at 50  $\mu\text{l}$  per well. After addition, the plates were allowed to stand for one hour at room temperature (primary antibody reaction). The  
5 reaction solution was then discarded and each well was washed four or more times with PBS. Anti-rabbit immunoglobulin antibody (secondary antibody) labeled with horseradish peroxidase (HRP) was then suitably diluted with PBS and added to each well in 50  $\mu\text{l}$  aliquots. This was then reacted for 30 to 60  
10 minutes at room temperature (secondary antibody reaction). Anti-rabbit IgG (H+L-chain) conjugated with peroxidase (MBL) was used as the secondary antibody. The secondary antibody reaction solution was then discarded and each well was washed four or more times with PBS. Coloring substrate solution (750  $\mu\text{M}$   
15 tetramethylbenzidine (TMB)) was then added to each well in 50  $\mu\text{l}$  aliquots, and color was developed for five to 20 minutes at 30°C (coloring reaction). The coloring reaction was stopped by adding 50  $\mu\text{l}$  aliquots of reaction stopping solution (1.5 N  $\text{H}_3\text{PO}_4$ ) to each well. Finally, optical absorbance at 450 nm was  
20 measured using a microplate reader.

Anti-Mcm2-phospho-S17 antibody was serially diluted in four-fold increments from 4  $\mu\text{g/ml}$  of antibody solution. Specificity was confirmed by ELISA using the plate on which phosphorylated peptide (Mcm2-phospho-S17) was immobilized, and  
25 the plate on which non-phosphorylated peptide (Mcm2-S17) was immobilized.

Fig. 2 shows the results of confirming the specificity of anti-Mcm2-phospho-S17 polyclonal antibody. As a result, the optical absorbance of the plate on which Mcm2-phospho-S17  
30 phosphorylated peptide was immobilized increased with increasing antibody concentration. The optical absorbance of the plate on which Mcm2-S17 non-phosphorylated peptide was immobilized remained at nearly zero. Thus, the subject antibody was proven to specifically recognize phosphorylation of the 17th serine  
35 residue of human Mcm2.

### Example 7 Construction of an ELISA System for Measuring Cdc7-ASK Complex Phosphorylation Activity

Regions comprising the 17th serine residue of human Mcm2, the substrate used, were selected in order to measure Cdc7-ASK complex phosphorylation activity in a 96-well microtiter plate. More specifically, the substrates studied were 1) GST fusion proteins with the amino acid regions of residues 1 to 130 and 1 to 80 of human MCM2, which phosphorylation assays using radioisotopes found to be a good substrate for Cdc7-ASK complex, and 2) a non-phosphorylated peptide corresponding to the amino acid sequence from the 10th to 20th amino acid residues (Mcm2-S17).

GST-Mcm2 (1-130 a.a.) and GST-Mcm2 (1-80 a.a.) were diluted to 5 µg/ml using 0.1 M carbonate buffer, pipetted at 50 µl/well in to each well of a microtiter plate for ELISA, and then sensitized overnight at 4°C. The sensitizing solution was removed, a blocking solution (1% BSA, 5% sucrose and 0.1% NaN<sub>3</sub>/PBS) was pipetted in to each well at 200 µl/well, and the plate was allowed to stand for about one hour at room temperature. The blocking solution was then completely removed. The plates were then air-dried in a draft chamber, and stored at 4°C until use. GST-Mcm2 (1-130 a.a.) and GST-Mcm2 (1-80 a.a.) were immobilized on the inner walls of the microtiter plates by this procedure.

A protein phosphorylation reaction was then carried out in the wells in which recombinant proteins GST-Mcm2 (1-130 a.a.), GST-Mcm2 (1-80 a.a.) or non-phosphorylated peptide (Mcm2-S17) had been immobilized. ELISA was then carried out in succession in the same wells.

A recombinant Cdc7-ASK complex dilution series was prepared by diluting from one-fold (x1) to 64-fold in two-fold increments using a phosphorylation buffer. 50 µl aliquots of this solution were added to the wells of the immobilized plates (a protein phosphorylation reaction). After incubating at 30°C for a fixed period of time, the phosphorylation reaction solution was discarded and each well was amply washed four or more times with

PBS. Primary antibody, that is, anti-Mcm2-phospho-S17 polyclonal antibody, was diluted with an antibody diluent (1% BSA, 0.1%  $\text{NaN}_3$ /PBS) to a concentration of 1  $\mu\text{g}/\text{ml}$ , added in 50  $\mu\text{l}$  aliquots to each well, and allowed to react for 30 to 60 minutes at room temperature (primary antibody reaction). The primary antibody reaction solution was then discarded and each well was amply washed four or more times with PBS. Anti-rabbit immunoglobulin antibody (secondary antibody, MBL) labeled with horseradish peroxidase (HRP) was then diluted 1000-fold with PBS and added to each well in 50  $\mu\text{l}$  aliquots, then allowed to react for 30 to 60 minutes at room temperature (secondary antibody reaction). The secondary antibody reaction solution was then discarded and each well was washed four or more times with PBS. A coloring substrate solution was then added to each well in 50  $\mu\text{l}$  aliquots, and color was developed for five to 20 minutes (coloring reaction). The coloring reaction was stopped by adding 50  $\mu\text{l}$  aliquots of reaction stopping solution to each well, and the optical absorbance at 450 nm was measured using a microplate reader.

The results are shown in Fig. 3. These results clearly demonstrate that the protein phosphorylation activity of a Cdc7-ASK complex active substance can be measured by ELISA, using the anti-phosphorylated peptide antibodies provided in the present invention. Recombinant protein GST-Mcm2 (1-130 a.a.) was determined to be the most preferable substrate for use in this phosphorylation activity measurement system. One reason why non-phosphorylated peptide (Mcm2-S17) cannot be used in this activity measurement system is presumed to be difficulty in Cdc7-ASK complex recognition of the target 17th serine residue, since the region comprising the phosphorylated site is short. Similar observations also suggest the possibility that GST-Mcm2 (10-20 a.a.) can also be provided as a substrate in the present measurement system.

Fig. 4 shows the results of investigating changes in reactivity when the final concentration of ATP added to the phosphorylation reaction solution was changed from 1  $\mu\text{M}$  to 2 mM,

in the method for measuring Cdc7-ASK complex activity using GST-Mcm2 (1-130 a.a.) and Mcm2-phospho-S17 polyclonal antibody, as described above. Enzyme activity is shown as a relative activity value (%), where activity at 2 mM is taken as 100. Activity of 90% is indicated at 0.1 mM, and the reaction virtually plateaus at 1 mM. Although activity equal to or more than 30% of the maximum activity value was indicated, even in the presence of low concentrations of ATP, reactivity was clearly shown to be ATP-dependent.

Fig. 5 shows the results of investigating changes in reactivity when the phosphorylation reaction time in the method for measuring Cdc7-ASK complex activity was changed from 0 to 150 minutes. Increases in absorbance were observed to be dependent on reaction time, and the reaction virtually plateaued at 90 minutes.

The use of other kinases in the present measurement system was examined. A GST-Mcm2 (1-130 a.a.)-sensitized plate and anti-Mcm2-phospho-S17 polyclonal antibody were used. The aforementioned Cdc7-ASK complex (wild type, WT) and inactive (KD) Cdc7-ASK complex were used as enzymes. Activity was measured for each dilution series.

The results are shown in Fig. 6. Optical absorbance, i.e., phosphorylation of the 17th serine residue, was observed to decrease as WT Cdc7-ASK complex was diluted by up to four-fold. This was not observed for KD Cdc7-ASK complex, in which phosphorylation activity is not present. On the basis of these findings, the present measurement system was proved able to measure Cdc7-ASK complex-specific phosphorylation activity.

A radiofilter assay is a method for detecting substrate phosphorylation that uses uptake of the radioisotope [ $\gamma$ - $^{32}$ P]ATP into an acid-insoluble fraction. It is frequently used in measuring phosphorylation activity. The sensitivity of this method and the ELISA measurement system provided in the present invention were compared.

A recombinant Cdc7-ASK complex dilution series diluted from one-fold (x1) to 64-fold in two-fold increments was prepared

using a phosphorylation buffer in 1  $\mu$ g of recombinant protein GST-Mcm2 (1-130 a.a.), and then adjusted to a total volume of 50  $\mu$ l. After incubating at 30°C for a fixed period of time, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid and 0.2%  $\text{Na}_4\text{P}_2\text{O}_7$ . The acid-insoluble fraction was trapped in a GFC filter (Whatman), and washed three times using 2% trichloroacetic acid and 0.02%  $\text{Na}_4\text{P}_2\text{O}_7$ . The radioactivity of the  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  taken up by the substrate was counted with a liquid scintillation counter.

The results are shown in Fig. 7. The results are shown as relative values (%), where both the radioactive count (cpm) in the radiofilter assay, and the optical absorbance in ELISA, are taken to be 100% at the one-fold concentration of Cdc7-ASK complex enzyme. Compared to the measurement system using radioisotope  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , the ELISA method could measure about 1/8 the amount of enzyme, since the value was 100% up to x8. In addition, while the amount of substrate used in the ELISA method is a calculated value of 0.25  $\mu$ g per well, the radiofilter assay requires about 1  $\mu$ g, or four times this amount. Furthermore, in the ELISA method there was hardly any background interference in the absence of enzyme. Thus, these systems are more sensitive, require smaller amounts of substrate and enzyme, and can be measured without using radioisotopes, demonstrating the superiority of the ELISA measurement systems provided by the present invention

K-252a (Calbiochem) and Staurosporine are known as kinase inhibitors. They are known as inhibitors of various kinases such as CaM kinase II, protein kinase A, protein kinase C, and protein kinase G. The ELISA systems provided by the present invention were studied using K-252a and Staurosporine. Dilution series of each inhibitor were prepared, mixed with Cdc7-ASK complex prepared with a phosphorylation assay buffer, and then adjusted to a total volume of 50  $\mu$ l. This was then added to wells in which GST-Mcm2 (1-130 a.a.) was immobilized. Using an above-described method, ELISA was then used to examine Cdc7-ASK complex phosphorylation activity.



The results are shown in Fig. 8. Fig. 8 shows the relative inhibition values (%), where the activity value without an inhibitor is taken as 100. Since both K-252a and Staurosporine inhibitors are kinase inhibitors with low specificity, inhibition of phosphorylation activity by Cdc7-ASK complex was concentration-dependent. The IC50 value was 2  $\mu$ M for both of these inhibitors. On the other hand, concentration-dependent inhibitory effect for Cdc7-ASK complex was not observed for Roscovitine, Olomoucine, and U0126. Roscovitine and Olomoucine are cdk2 inhibitors, and U0126 is a MAP kinase inhibitor. These kinase inhibitors were shown to lack Cdc7-ASK complex inhibition activity. Therefore, the ELISA system provided by the present invention was in fact shown to be effective in screening for agents that inhibit Cdc7-ASK complex phosphorylation activity.

#### Industrial Applicability

According to the present invention, the mechanism by which the substrate protein, MCM2, is phosphorylated by Cdc7-ASK complex was elucidated. On the basis of this, substrate protein phosphorylation by the Cdc7-ASK complex could be more specifically evaluated. In other words, the methods for measuring phosphorylation effect of the present invention are useful as methods in which the phosphorylating action of Cdc7-ASK complex can be specifically evaluated. For example, the kinase activity of Cdc7-ASK has been clearly shown to increase in actively growing cancer cells (Gene, 1998, Apr 28;211(1):133-40, "A human homolog of the yeast CDC7 gene is overexpressed in some tumors and transformed cell lines.", Hess G.F., Drong R.F., Weiland K.L., Slightom J.L., Sclafani R.A., and Hollingsworth R.E., Cancer Research, Pharmacia, Upjohn, Inc., 301 Henrietta Street, Kalamazoo, MI 49001, USA). Thus, the growth ability of certain cancer cells can be predicted based on the kinase activity of Cdc7-ASK. In addition, by comparing the amount of expressed Cdc7 in numerous human cancer cells and normal cells, the present inventors also confirmed that Cdc7 is extremely strongly expressed in nearly all cultured cancer cells

(including fibroblast cell lines and lymphoid lines). Thus, Cdc7-ASK kinase activity, which can be evaluated by the methods of the present invention, is useful as a carcinogenesis marker. Cdc7-ASK kinase activity can be specifically evaluated by the present invention. Thus, the growth ability of cancer cells can be evaluated more specifically.

Moreover, by applying the aforementioned measurement methods, the present invention has realized methods for evaluating the effects of test compounds on the phosphorylation function of the Cdc7-ASK complex, as well as screening methods based on these evaluation methods.

The present invention's methods for measuring Cdc7-ASK kinase activity, or of screening for compounds that regulate that activity, are useful for the development of cancer medicaments and treatment methods targeting Cdc7-ASK. Compared to using known intracellular kinases (such as Cdk-Cyclin) or proteins as targets, the use of Cdc7-ASK as a target offers the following advantages:

Firstly, by targeting Cdc7-ASK, more reliable control of activity can be expected. While Cdk-Cyclin is a family composed by numerous similar genes, Cdc7-ASK is composed of molecules whose structure is more limited. Thus, by using Cdc7-ASK as a target, activity can be reliably controlled.

Secondly, using Cdc7-ASK as a target makes it possible to specifically control cell growth. Cdc7-ASK is a factor required for the initiation and progress of the S phase. Upon loss of this activity the S phase immediately stops and cell growth is arrested. Moreover, the results of experiments using genetically manipulated mice suggest that interruption of the S phase, namely DNA replication, can potentially cause abnormal structures to accumulate in DNA, which when detected as DNA damage, can induce p53 and eventually cell death. In other words, inhibition of Cdc7-ASK activity can effectively block progression of the S phase in cancer cells, efficiently removing cancer cells by inducing cell death.

Thirdly, the use of Cdc7-ASK as a target is expected to

lead to the discovery of completely new cell growth inhibitors. The structure of Cdc7-ASK kinase is unique, even among members of the kinase family. In addition, the structure of ASK is also different from that of cyclin molecules, on which considerable  
5 research has been conducted. This activation is predicted to be due to a novel, and heretofore unknown, mechanism regarding kinase activation. Thus, searching for activity inhibitors that target Cdc7-ASK is highly likely to lead to the discovery of completely new substances, undiscovered by the various types of  
10 kinase screening to date. This means that, for example, new lead compounds may be discovered in the research and development of anti-cancer agents.

Fourthly, induction of cell death can be expected when using Cdc7-ASK as a target. In response to the unexpected  
15 arrest of DNA replication, cells activate so-called check-point kinases, such as ATM, Chk1, and Cds1, which respond by delaying the progress of the cell cycle. If Cdc7-ASK inhibitors are used in combination with inhibitors against these check-point kinases, enhanced induction of cell death, due to inhibited S phase  
20 progression caused by inhibiting Cdc7-ASK activity, can be expected.

In this manner, Cdc7-ASK complex plays important roles in the initial stages of cell growth in the cell cycle. Moreover, its phosphorylation activity is proportional to the ability of  
25 cancer cells to grow. In other words, cancer cell growth can be more specifically controlled by controlling Cdc7-ASK complex activity. Thus, the screening methods of the present invention are useful as methods for obtaining compounds that can specifically control cancer cell growth.

30 Moreover, the screening methods of the present invention are also able to more specifically evaluate the effects of test compounds on the phosphorylation function of Cdc7-ASK complex. As a result, compounds that act more specifically on cancer can be selected.

35 In addition, the present invention has also provided a methods for preparing the Cdc7-ASK complex active substances

required by each of the aforementioned methods. According to the methods of the present invention, complexes comprising activity similar to that of the Cdc7-ASK complex can be easily prepared in large volumes by using prokaryotic cells.

5 Furthermore, the present invention provides substrate proteins that are phosphorylated by the Cdc7-ASK complex. The substrate proteins of the present invention are proteins that comprise the structure required for phosphorylation by the Cdc7-ASK complex (or Cdc7-ASK complex active substance). By using  
10 such substrate proteins the phosphorylation effect of Cdc7-ASK complex can be more specifically evaluated. The Cdc7-ASK complex active substances and substrate proteins prepared based on the present invention are useful in the aforementioned methods.

15 In addition, the present invention has provided antibodies that identify the level of phosphorylation of substrate proteins phosphorylated by the Cdc7-ASK complex or Cdc7-ASK complex active substance. The antibodies of the present invention identify the level of phosphorylation at specific substrate  
20 protein sites. These antibodies make it possible to more specifically evaluate the phosphorylation effect of the Cdc7-ASK complex or Cdc7-ASK complex active substance. In addition, the use of the antibody of the present invention enables the phosphorylation effect of the Cdc7-ASK complex or Cdc7-ASK  
25 complex active substance to be easily evaluated based on immunoassay principles.